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The Microneme Proteins EtMIC4 and EtMIC5 of *Eimeria tenella* Form a Novel, Ultra-high Molecular Mass Protein Complex That Binds Target Host Cells*

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***Eimeria tenella*, in common with other parasitic protozoa of the phylum Apicomplexa, invades host cells using an actinomyosin-powered "glideosome" complex and requires the secretion of adhesive proteins from the microneme organelles onto the parasite surface. Microneme proteins of *E. tenella* include EtMIC4, a transmembrane protein that has multiple thrombospondin type I domains and calcium-binding epidermal growth factor-like domains in its extracellular domain, and EtMIC5, a soluble protein composed of 11 tandemly repeated domains that belong to the plasminogen-apple-nematode superfamily. We show here that EtMIC4 and EtMIC5 interact to form an oligomeric, ultrahigh molecular mass protein complex. The complex was purified from lysed parasites by non-denaturing techniques, and the stoichiometry was shown to be $[EtMIC4]_2$:$[EtMIC5]_1$, with an octamer of EtMIC4 bound non-covalently to a tetramer of EtMIC5. The complex is formed within the parasite secretory pathway and is maintained after secretion onto the surface of the parasite. The purified complex binds to a number of epithelial cell lines in culture. Identification and characterization of this complex contributes to an overall understanding of the role of multimolecular protein complexes in specific interactions between pathogens and their hosts during infection.**

Parasitic protozoa of the phylum Apicomplexa include many pathogens of humans and animals such as *Plasmodium* spp. that cause malaria, *Toxoplasma gondii* that causes abortion and encephalitis, and *Eimeria* spp. that cause severe enteritis (coccidiosis). Apicomplexans are obligate, intracellular pathogens that glide and actively invade host cells using a specialized actinomyosin-powered "glideosome" complex (1) that lies within the trilaminar pellicle of the parasites (2–4). Invasion is regulated by calcium-dependent signaling pathways in the parasite (5) with apical contact of the parasite to the host cell signaling the release of proteins from the parasite secretory microneme organelles onto the parasite surface (6). Successful gliding and invasion requires the timely engagement and disengagement of these surface-exposed, microneme-derived receptors with host cell ligands (7, 8) coupled to the processive, rearward capping of the receptor-ligand complexes over the whole length of the parasite surface (9–12).

Micronemes contain mixtures of soluble and transmembrane proteins (MICs),[4] many of which bear multiple copies of a limited number of adhesive protein domains (13). Oligomerization of MICs to form functional adhesive complexes has been demonstrated in a number of apicomplexans, and typically, these contain a single transmembrane (TM) "escorter" MIC tightly associated with one or more soluble "cargo" MICs. A crucial function of oligomerization is correct and cooperative targeting of proteins through the secretory pathway to the micronemes. Genetic disruption of an escorter MIC results in incorrect localization of all its associated cargo (14–18), and tyrosine-based sorting motifs, capable of redirecting the trafficking of a surface-expressed protein, TgSAG1, to the micronemes, were precisely defined within the C-terminal cytosolic tail of the TM MIC TgMIC2 (19). The cargo MICs are also important in trafficking and may be crucial for correct aggregation or folding of complexes; for example, the galectin domain of soluble TgMIC1 promotes and stabilizes the oligomerization and folding of TgMIC1-4-6 complex that is essential for the exit of this complex from the Golgi (20). In contrast, a short N-terminal pro-peptide of soluble TgM2AP is essential for the trafficking of TgMIC2-M2AP complex through a post-Golgi, endosomal-related compartment (21).

Once an MIC complex is exposed on the parasite surface, the C-terminal cytosolic tails of the TM MICs play another essential role in linking the complex to the underlying glideosome through interaction with a tetramer of 1,6-biphosphate aldolase, which in turn binds actin (16, 22). In *Plasmodium berghei*, the interaction of aldolase with the microneme protein PbTRAP is dependent on both a subterminal tryptophan in the PbTRAP tail and charge attraction between acidic residues in the tail and basic residues in the aldolase (22).

MIC complexes have been characterized mainly by genetic manipulation in *T. gondii* and *P. berghei*; however, oligomerization is likely to be conserved in all Apicomplexa. For example,

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[4] The abbreviations used are: MIC, microneme protein; M2AP, MIC2-associated protein; TM, transmembrane; Et, *E. tenella*; Tg, *T. gondii*; Pb, *P. berghei*; TSR, thrombospondin type I sequence repeat; EGF, epidermal growth factor; MDBK, Madin-Darby bovine kidney; PBS, phosphate-buffered saline; PAN, plasminogen-apple-nematode.

Eimeria tenella EtMIC1 and EtMIC2 are direct homologues of TgMIC2 and TgM2AP, and they form a complex homologous to that of TgMIC2-Tg2AP (23). Moreover, complementation studies in *T. gondii* show that expression of EtMIC1–2 permits the knock-out of the essential endogenous gene, *TgMIC2* (24). Studies to characterize additional apicomplexan MIC complexes await identification of novel MICs, and among those already described in *E. tenella* are EtMIC4, a 240-kDa TM MIC with 16 thrombospondin type I (TSR) domains and 31 epidermal growth factor-like (EGF) domains (25, 26), and EtMIC5, a 100-kDa soluble MIC with 11 domains that belong to the plasminogen-apple-nematode (PAN) superfamily (27, 28). We show now that these MICs associate to form an ultrahigh molecular mass oligomeric complex. Analytical size exclusion chromatography indicates that the complex has a stoichiometry of $[EtMIC4]_2$:$[EtMIC5]_1$ and that an octamer of EtMIC4 binds non-covalently to a tetramer of EtMIC5. The complex has binding activity in cell-based assays, indicating that it likely functions as a parasite receptor for a host cell ligand during the process of parasite invasion of host cells.

EXPERIMENTAL PROCEDURES

Immunoprecipitation—The method is based on that of Ref. 29. Briefly, 1.8×10^6 Madin-Darby bovine kidney (MDBK) cells infected with 25×10^6 freshly excysted sporozoites of *E. tenella* (30) were lysed in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 100 mM NaCl, 5 mM EDTA) and clarified by centrifugation at 10,000 × *g* for 3 min. To the supernatant, 100 μl of 10% protein A beads (Sepharose CL-4B, Sigma) were added, the contents were mixed end-over-end for 1 h at 4 °C, and the beads were removed by centrifugation at 10,000 × *g* for 15 s. Supernatant was incubated with 5 μl of normal rabbit serum for 1 h on ice, 200 μl of protein A beads were added and removed as before, and then the supernatant was incubated with 30 μl of an affinity-purified rabbit anti-EtMIC5 serum (RbαEtA9, 0.5 mg ml^{-1}, Brown *et al.* (27)) for 1 h on ice, and a final 200 μl of protein A beads were added and removed, as before. Protein A pellets were washed three times in radioimmunoprecipitation assay buffer and denatured by heating to 85 °C for 10 min, and proteins were analyzed by SDS-PAGE followed by silver staining or Western blotting. Sera used to probe Western blots were hyperimmune rabbit anti-microneme (RbαMIC (12)), hyperimmune chicken anti-EtMIC5 (ChαEtMP (27)), and hyperimmune rabbit anti-EtMIC4 (RbαEtMIC4 (25)).

Preparation of Oocyst Protein Extract—*E. tenella* (Houghton strain) oocysts were propagated, recovered, and sporulated following standard procedures (30). A culture of 10^9 fully sporulated oocysts was pelleted by centrifugation, suspended in 50 ml of 40 mM Tris buffer, pH 8, containing EDTA-free protease inhibitors (Roche Applied Science) at the manufacturer's recommended dilution, and broken to completion by vortexing with glass beads as described previously (31). The liquid over the beads was harvested, subjected to three rounds of freeze-thawing, cleared by centrifugation at 5000 × *g* for 5 min, and sonicated for three bouts of 20 s at 10-μm amplitude. DNase I (0.2 mg ml^{-1} Invitrogen) was added, and the mixture was incubated for 1 h at 37 °C followed by centrifugation at 5000 × *g* for 5 min, filtration of the supernatant through a 2.5-μm filter, and storage at −80 °C until use.

Semipreparative Anion Exchange and Size Exclusion Chromatography—The purification strategy was adapted from that of Ref. 32. Oocyst protein extract was loaded onto a home-packed anion exchange column (Q Sepharose, Amersham Biosciences) with a 35-ml bed volume at a flow rate of 3 ml min^{-1}. Bound components were washed with 40 mM Tris, pH 8.0, and eluted in a linear 0–1 M NaCl gradient in the same buffer. Fractions of 5 ml were collected and were assayed for EtMIC4 and EtMIC5 by SDS-PAGE followed by silver staining or Western blotting. Fractions containing target proteins were pooled, concentrated to ~2 ml through a 30-kDa cut-off filter (Amicon), and subjected to semipreparative size exclusion chromatography. Briefly, a home-packed column of Sephacryl S-300 (Amersham Biosciences, 16-mm diameter, 55-cm length) was pre-equilibrated with 100 mM NaCl, 40 mM Tris-HCl, pH 8, and injected at a flow rate of 0.8 ml min^{-1}. Fractions of 5 ml were collected and assayed as above.

Analytical Size Exclusion Chromatography—Fractions from the semipreparative size exclusion column containing EtMIC4 and EtMIC5 were pooled, concentrated through a 30-kDa cut-off filter (Amicon) pretreated with bovine serum albumin to reduce nonspecific adsorption, and reanalyzed by use of an analytical size exclusion column (Superose 6PC 3.2/30, Amersham Biosciences, making use of a, Amersham Biosciences Smart machine). The column was pre-equilibrated in 100 mM NaCl, 40 mM Tris-HCl, pH 8, and injected at a flow rate of 40 μl min^{-1}, and 100-μl fractions were collected and assayed for EtMIC4 and EtMIC5 by SDS-PAGE followed by silver staining or Western blotting. The analytical column was calibrated with a series of individual runs of standard molecular mass markers (Amersham Biosciences) dissolved at 10 μg μl^{-1}: monomeric and dimeric thyroglobulin (669-kDa monomer), apoferritin (443-kDa monomer), and albumin (66-kDa monomer).

To investigate the composition of the EtMIC4–5 protein complex in more detail, a portion of the material recovered from the analytical column was denatured in 6 M guanidinium hydrochloride for 24 h at 37 °C and then reanalyzed. In this case, the column was pre-equilibrated in 100 mM NaCl, 40 mM Tris-HCl, pH 8, containing 6 M guanidinium hydrochloride and 50-μl fractions assayed as above.

Cell Lines—Cell lines were propagated in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, 1.2 mg ml^{-1} sodium bicarbonate, 0.4 mg ml^{-1} G418, 10% fetal calf serum.

Cell Binding Assays—Binding assays were carried out using proteins derived from broken oocysts, purified sporozoites and purified microneme organelles (33) at stock concentrations of ~5 μg ml^{-1} protein. Before use, all samples were subjected to three rounds of freeze-thawing followed by three bouts of sonication at 10 μm amplitude for 20 s and centrifugation for 1 min at 10,000 × *g* in a microcentrifuge. 50 μl of supernatant were mixed with 200 μl of phosphate-buffered saline (PBS) and used for cell binding assays following a previously described protocol (34). Briefly, confluent monolayers in 6-well plates were washed

with PBS, blocked by incubation in PBS, 1% bovine serum albumin for 2 h at 4 °C, rinsed three times with PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and then incubated in the diluted protein solution also containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ for 1 h. Unbound supernatant was removed, monolayers were washed five times in PBS, and monolayers were then harvested by scraping. All samples (sonicated input sample, unbound fraction, final wash, and cell bound fraction) were analyzed by reducing SDS-PAGE and Western blotting.

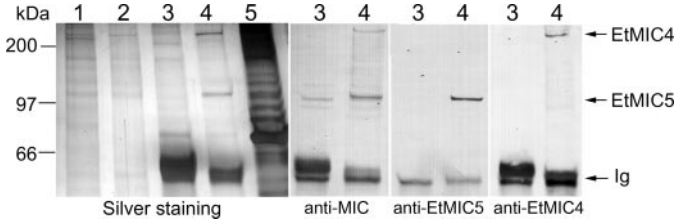

FIGURE 1. **EtMIC4 and EtMIC5 proteins are co-precipitated by antibodies to EtMIC5.** Proteins were precipitated from a lysate of *E. tenella* sporozoite-infected cells, and samples were examined by reducing SDS-PAGE followed by silver staining and Western blotting with rabbit anti-microneme (*anti-MIC*), chicken anti-EtMIC5 (*anti-EtMIC5*), or rabbit anti-EtMIC4 (*anti-EtMIC4*) sera. *Sample 1*, insoluble material pelleted after lysis; *sample 2*, precipitate formed by preclearance of lysate with protein A beads; *sample 3*, precipitate formed using normal rabbit serum; *sample 4*, precipitate formed using affinity-purified rabbit anti-EtMIC5 serum (RbαEtA9); *sample 5*, remaining soluble lysate.

RESULTS

Co-immunoprecipitation of EtMIC4 and EtMIC5—During invasion of host cells, EtMIC5 is distributed in a punctuate pattern over the surface of invading zoites (35) in a manner reminiscent of the distribution of TgMIC2-M2AP (36), TgMIC1-4-6 (14), and EtMIC1–2 (12). Since EtMIC5 is a soluble MIC, we hypothesized that its surface tethering and distribution are mediated by interaction with another, transmembrane, MIC. To investigate this possibility, affinity-purified antibodies derived from rabbit hyperimmune serum against a single, recombinant-expressed Apple domain of EtMIC5 were used in immunoprecipitation experiments (Fig. 1). In all experiments, whether using lysates of sporozoites alone or lysates of sporozoite-infected MDBK epithelial cells, two independent polypeptide species were precipitated. One, which migrated under reducing SDS-PAGE conditions as a single ~100-kDa band, was identified by Western blotting as EtMIC5, and the other, a single ~240-kDa band, was identified as EtMIC4, a multimodular, transmembrane MIC protein also under study in our laboratory (25, 26). Silver staining of the precipitated material did not identify any additional proteins pulled down specifically by the antibodies to EtMIC5, indicating that EtMIC4 and EtMIC5 most likely form a two-partner protein complex.

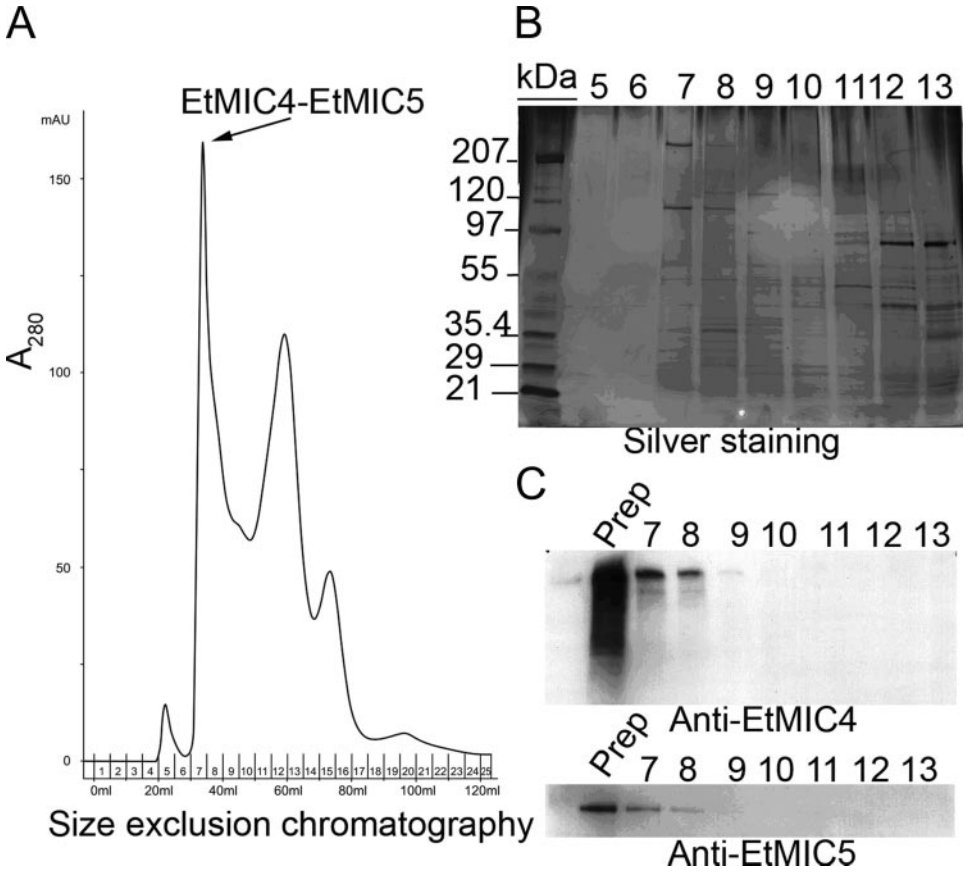

FIGURE 2. **EtMIC4 and EtMIC5 proteins co-purify by semipreparative size exclusion chromatography.** *A*, parasite proteins derived from sporulated oocysts were separated by anion exchange chromatography, and fractions containing EtMIC4 and EtMIC5 were pooled, concentrated, and loaded onto a 60 × 16-cm Sephacryl 300 (Amersham Biosciences) column. EtMIC4 and EtMIC5 elute in fractions 7 and 8, which correspond to the size exclusion limit (>1.5 mDa) of the column. *B*, proteins of the expected mass of monomers of EtMIC4 and EtMIC5 were detected in fractions 7 and 8 by reducing SDS-PAGE and silver staining. *C*, the identities of proteins in fractions 7 and 8 as EtMIC4 and EtMIC5 were confirmed by probing Western blots with polyclonal antisera against EtMIC4 (RbαEtMIC4) and EtMIC5 (RbαEtA9). *Prep* = whole protein preparation; *numbers* correspond to elution fractions. Elution fractions = 5 ml.

Purification of High Molecular Mass EtMIC4–5 Oligomers—For independent verification that there is a biological interaction between EtMIC4 and EtMIC5, a protein purification strategy was applied to fully sporulated parasite oocysts, which contain mature sporozoites and, therefore, micronemes. The protocol makes use of the relatively low predicted pI (4.2) of the EtMIC4–5 complex to gain a significant first step purification by anion exchange chromatography (32) followed by preparative and analytical size exclusion chromatography. The protocol did not contain detergent since it has been demonstrated already for a number of transmembrane MICs that they are soluble in the absence of detergent (15, 23), and it was expected that this non-denaturing protocol would deliver purified protein in a near native conformation.

After anion exchange chromatography at pH 8.0, fractions con-

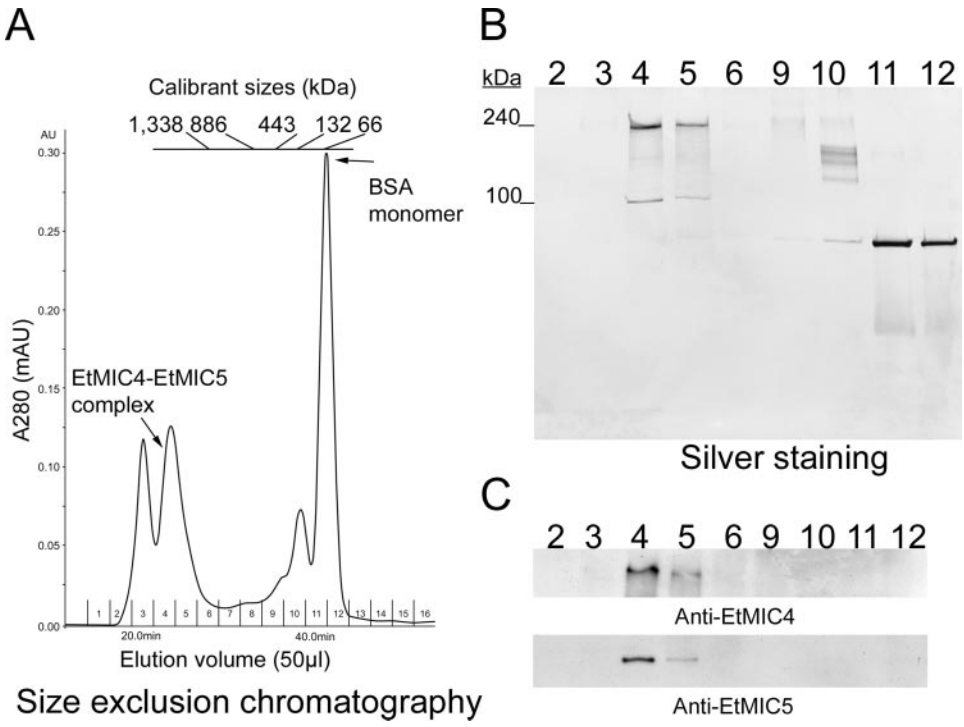

FIGURE 3. **Purification of the EtMIC4–5 protein complex by analytical size exclusion chromatography.** *A*, concentrated material from fractions 7 and 8 of a semipreparative size exclusion column was pooled, concentrated, and loaded onto an Amersham Biosciences Superose 6PC 3.2/30 analytical column. EtMIC4 and EtMIC5 co-elute, in fractions 4 and 5, which are estimated to contain proteins of ~2 MDa in mass. *mAU*, milliabsorbance units; *BSA*, bovine serum albumin. *B*, proteins of the expected mass of monomers of EtMIC4 and EtMIC5 were detected in fractions 4 and 5 by reducing SDS-PAGE and silver staining. *C*, the identity of proteins in fractions 4 and 5 as EtMIC4 and EtMIC5 were confirmed in Western blots. Elution fractions = 100 µl.

taining EtMIC4 and EtMIC5 (monitored by reducing SDS-PAGE and Western blotting) eluted at around 400 mM salt (data not shown) and were pooled, concentrated, and further purified by use of a home-packed semipreparative size exclusion column. Fractions eluting from this column were screened by reducing SDS-PAGE followed by silver staining and Western blotting with antibodies against EtMIC4 and EtMIC5. Both proteins were found exclusively in two early eluting fractions, 7 and 8, at the size exclusion limit of the column, which is in excess of 1.5 MDa (Fig. 2, *A–C*). This strongly suggests that EtMIC4 and EtMIC5 exist within the parasite as a high molecular weight complex. Silver staining revealed that as well as the two dominant EtMIC4 and EtMIC5 protein species, there are other, less abundant, proteins present in fractions 7 and 8. Following additional purification over an analytical size exclusion column, capable of resolving very high molecular mass protein species (Fig. 3), these contaminants were found not to co-purify with EtMIC4 and EtMIC5. The first elution peak from the analytical column (fraction 3) contains material that is larger than the exclusion limit and is not retained on the column. This did not stain with silver (Fig. 3*B*), nor did it react with antibodies to EtMIC4 or EtMIC5 after reducing SDS-PAGE (Fig. 3*C*). The second peak (fractions 4 and 5) with an apparent molecular mass in excess of 2 MDa contains solely EtMIC4 and EtMIC5 (Fig. 3, *B* and *C*). Peaks eluting later did not contain any EtMIC4 or EtMIC5 but did contain some unidentified proteins and bovine serum albumin, which had been used to block adsorption of proteins to the concentration filters. The chromatographic co-purification of EtMIC4 and EtMIC5 from the native parasite lysate fully supports the co-immunoprecipitation data and suggests strongly that they exist as a very high molecular mass, multimeric complex that is in excess of 2 MDa.

The EtMIC4-EtMIC5 Complex Consists of Two Homomultimers—To investigate further the EtMIC4–5 protein complex, portions of fractions 4 and 5 from the analytical size exclusion column were pooled, concentrated, incubated with 6 M guanidinium hydrochloride at 37 °C overnight, and then reanalyzed on the same analytical size exclusion column. Under these strongly denaturing but non-reducing conditions, the EtMIC4–5 complex dissociated into two well resolved peaks (Fig. 4*B*), in contrast to a control sample that had not been treated with guanidinium hydrochloride (Fig. 4*A*). When subjected to reducing SDS-PAGE, the first peak (fractions 9–12), with a chromatographic molecular mass in excess of 1.5 MDa, was shown to be composed entirely of EtMIC4, and the second peak (fractions 18–20), with a chromatographic molecular mass of ~400 kDa, was composed entirely of EtMIC5 (Fig. 4*C*). These data suggest that the EtMIC4–5 complex comprises two discrete homomultimers of EtMIC4 and EtMIC5 linked by non-covalent interactions that are dissociated by denaturation. Since incubation in 6 M guanidinium hydrochloride did not yield any evidence of disruption or breakage of the individual homomultimers, another portion of fractions 4 and 5 of the analytical size exclusion column was disrupted in 6 M guanidine supplemented with β-mercaptoethanol and dithiothreitol and then reanalyzed. Under these conditions, a complex chromatogram was obtained (data not shown) in which EtMIC4 and EtMIC5 were distributed over peaks corresponding in mass to monomers, homodimers, and homotetramers. From this, we conclude that the overall complex is indeed formed by the non-covalent interaction of homomultimers of EtMIC4 and EtMIC5 and that these can be at least partially denatured under reducing conditions. All of the cysteine residues present in the extracellular domains of EtMIC4 and EtMIC5 are predicted to form intramolecular disulfide bridges within TSP-1, EGF, or Apple domains, and there is no evidence for intermolecular disulfide bridging between monomers.

Integration of the EtMIC4 and EtMIC5 peaks in UV280 chromatograms such as that shown in Fig. 4*B* was used to calculate their concentrations, relative to each other, by comparison of UV absorbance with the theoretically calculated extinction coefficients of each species. This resulted in calculated relative concentrations, and therefore, stoichiometry, of almost

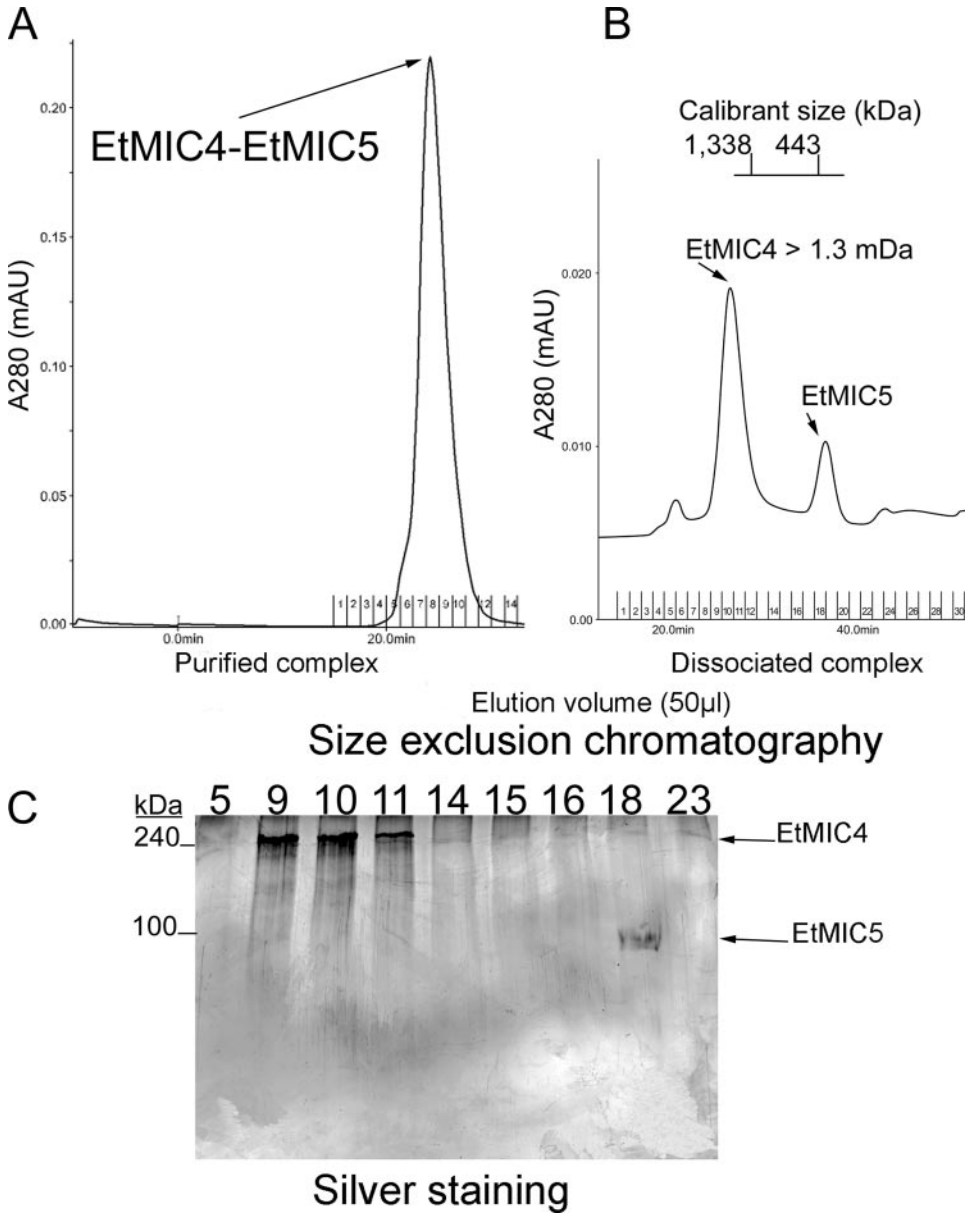

FIGURE 4. **Chemical denaturation of the EtMIC4–5 protein complex.** *A*, in the absence of denaturation, the EtMIC4-EtMIC5 protein complex repurifies over the Amersham Biosciences Superose 6PC column as a single peak. *mAU*, milliabsorbance units. *B*, denaturation of this material by incubation in 6 M guanidium hydrochloride at 37 °C for 24 h results in a shift of the elution profile to two peaks, one of >1.3 mDa and a second one of ~450 kDa. *C*, SDS-PAGE and silver staining confirmed that the >1.3-mDa peak (fractions 9–11) contains only EtMIC4, whereas the 450-kDa peak (fraction 18) contains only EtMIC5. Elution fractions = 50 µl.

exactly 2:1 EtMIC4:EtMIC5 (Table 1). Elution volumes of the two peaks, in relation to those of standard calibrants eluted on the same column, suggest that the overall composition of the complex is most likely to be $[EtMIC4]_8[EtMIC5]_4$.

All of the above work was carried out on proteins isolated from broken oocysts within which microneme proteins are stored, most likely in an inactive form, within the apical microneme organelles that are part of the elaborated secretory pathway of the encysted parasite. Upon excystation and exposure of motile sporozoites to host cells, microneme secretion occurs rapidly (11, 12), and during invasion, MICs are capped over the parasite surface and released into the surrounding environment by proteolytic cleavage within a conserved TM domain of TM MICs (37). This TM domain is present in EtMIC4 (25), and the protein is released from the parasite membrane at invasion by proteolytic cleavage (data not shown). To examine whether EtMIC4–5 is maintained as a high molecular mass complex during and after invasion, we examined the chromatographic behavior of the proteins within excreted-secreted material harvested from the tissue culture supernatant after sporozoite infection of MDBK epithelial cells. The quantity of protein available for this analysis was considerably less than for total parasite lysate, but nonetheless, it is clear that the majority of EtMIC4 and EtMIC5 co-elute as a complex of a similar molecular mass

TABLE 1
Stoichiometry of the EtMIC4:EtMIC5 complex

Protein	EtMIC4		EtMIC5	
	No. in sequence[a]	Contribution to ϵ_{280}	No. in sequence[a]	Contribution to ϵ_{280}
Trp[b]	24	136,560	9	51,210
Tyr[b]	37	47,360	24	30,720
Cys[b]	249	29,880	66	7920
ϵ_{280}	213,800		89,850	
Peak area, arbitrary units[c]	57.38		12.36	
Relative No. of moles ($\times 10^{-6}$)[d]	268		138	
Stoichiometry	1.94:1			

[a] Number of Trp, Tyr, and Cys residues were determined after removal of N-terminal signal peptides and, in the case of EtMIC5 the propeptide (Brown *et al.* (35)).
[b] Values for extinction coefficients are 5690 M^{-1} cm^{-1} for each tryptophan residue, 1280 M^{-1} cm^{-1} for each tyrosine residue and 120 M^{-1} cm^{-1} for each cysteine residue (Gill and von Hippel (49)).
[c] Peak areas calculated by integration of the chromatogram shown in Fig 4*B*.
[d] Relative number of moles present in each peak calculated by dividing peak area by the extinction coefficient.

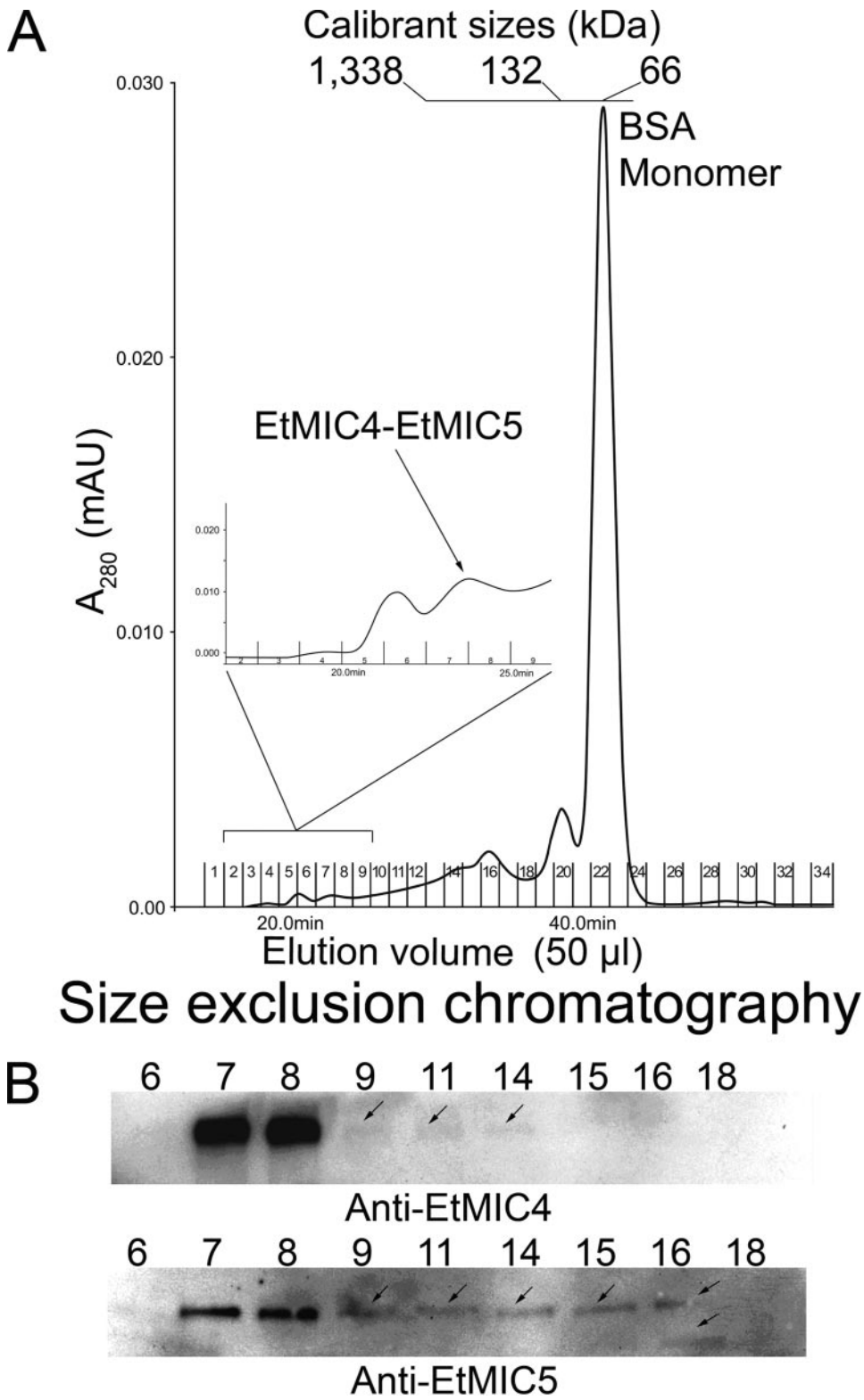

FIGURE 5. **EtMIC4–5 is secreted as a protein complex by the motile sporozoite stage of *E. tenella*.** *A*, excreted-secreted proteins were harvested from *in vitro* cultured sporozoites, and after one round of anion exchange chromatography, they were analyzed by analytical size exclusion chromatography over the Amersham Biosciences Superose 6PC column. The EtMIC4-EtMIC5 protein complex eluted in fractions that correspond to the same molecular mass as those in which the complex isolated from oocysts eluted (*e.g.* Fig. 4*A*). *mAU*, milliabsorbance units. *B*, SDS-PAGE and Western blotting confirmed that EtMIC4 and EtMIC5 are found predominantly in fractions 7–8, but additional signals were also detected in lower molecular mass elution fractions. Elution fractions = 50 μl.

to that observed for the smashed oocyst material (Fig. 5, *A* and *B*, *fractions 7* and *8*), This suggests that the complex is maintained during and after invasion in broadly the same composition as it is found within the parasite secretory pathway. However, EtMIC4 and EtMIC5 were also detected, albeit in significantly lower amounts, in elution fractions corresponding to smaller molecular masses (Fig. 5*B*, *fractions 9–16*), suggesting that the complex is less stable after its release from the sporozoite into the external environment.

EtMIC4–5 Protein Complex Binds to Cell Lines—The predicted amino acid sequences of EtMIC4 and EtMIC5, which include multiple TSR, EGF, and Apple/PAN domains, suggest that they may have a role in mediating parasite attachment to host cells. To investigate this, we used a simple assay to detect binding of exogenous protein to cultured cells that has been used for testing adhesive properties of proteins from other parasites (34). Assays were performed at 4 °C to avoid nonspecific uptake of exogenous proteins by endocytosis. Initially, lysates of proteins derived from broken oocysts were tested, but no convincing binding of EtMIC4 or EtMIC5 was consistently detected (data not shown). We reasoned that this may be because EtMIC4–5 is at (relatively) low abundance within the lysates, making detection in cell-bound fractions beyond the limit of the assay. When binding assays were carried out with solubilized, semipurified microneme organelles, isolated from disrupted *E. tenella* sporozoites by sucrose-gradient ultracentrifugation (33) or with the purified EtMIC4–5 complex eluted from the analytical size exclusion column, binding of both partners was detected across a wide range of cell lines (for example, Fig. 6).

DISCUSSION

The formation of complexes between soluble and transmembrane MICs provides a mechanism

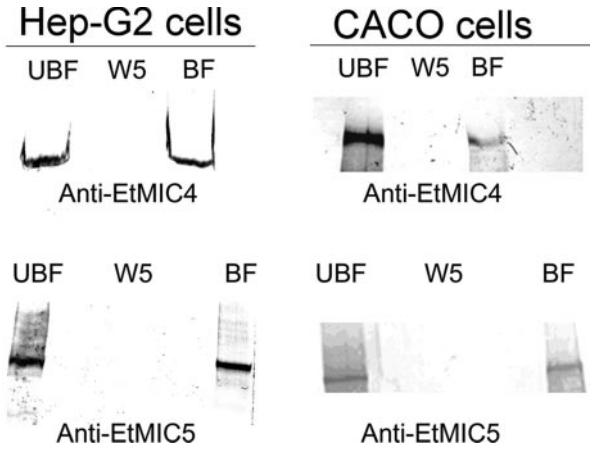

FIGURE 6. **EtMIC4–5 protein complex binds cultured cells.** Protein lysates solubilized from semipurified microneme organelles were incubated with cultured cells for 1 h at 4 °C, and then unbound material was removed by extensive washing, and samples were analyzed by SDS-PAGE and Western blotting. *UBF*, unbound fraction after 1 h of incubation; *W5*, fifth (final) wash of the cells; *CBF*, cell-bound fraction. All samples were processed in the same volumes.

whereby transmembrane and soluble proteins cooperatively reach the target organelle (15), utilizing sorting signals contained within the cytosolic tail of the transmembrane partner (19) and requiring the active participation of soluble cargo for correct folding and trafficking (14, 20, 21). EtMIC4 and EtMIC5 of *E. tenella* form a novel, ultrahigh molecular mass protein complex that exists in a native form at >2 Mda. Disassociation of the complex under denaturing conditions indicates that it is composed of homomultimers of EtMIC4 and EtMIC5 that are individually stabilized by disulfide bridging and held together by non-covalent interactions. The overall composition of the complex is calculated to be most likely $[EtMIC4]_8[EtMIC5]_4$, implying that octamers and tetramers of EtMIC4 and EtMIC5, respectively, assemble *in vivo* prior to heterocomplex formation. This is similar in principle to the proposed formation of an MIC complex in the related apicomplexan parasite *T. gondii* where TgMIC2 and TgM2AP exist as a functional hexameric complex composed of trimers of each partner that associate in a 1:1 ratio; when the *TgM2AP* gene is ablated, TgMIC2 still forms trimers (17).

The precise reasons for oligomerization and heterocomplex formation between EtMIC4 and EtMIC5 are unknown, but several parallels may be drawn with other secreted proteins, including those from parasites and higher eukaryotes. Oligomerization may increase avidity of the cytosolic tails of EtMIC4 for sorting receptors in adaptor protein complexes of the secretory pathway, some of which are known to be conserved in apicomplexans (38) since it is known that these interactions have low affinities when compared with other protein-protein interactions (39). Protein sorting to regulated secretion organelles, such as to the secretory granules of endocrine and neuroendocrine cells, is also dependent on the interaction between transmembrane proteins bearing sorting signals in their cytoplasmic tails and cargo proteins. Oligomerization changes the aggregation state of proteins within the trans-Golgi network and immature secretory granules and allows segregation of regulated secretion vesicles from those that are constitutively secreted (40). This form of selective sorting, mediated by aggregation, occurs upon exposure to the mildly acidic and high calcium environments encountered in the trans-Golgi network (41) and within immature secretory granules (42). EtMIC4 is an acidic protein (pI 3.9) that has 31 EGF-like domains, most of which can bind calcium (26). Oligomerization and complex formation of EtMIC4 with EtMIC5 (pI 6.3) in the parasite late secretory pathway may modify its aggregation/multimerization/folding state. A role for the endosomal system in sorting and packaging of secretory proteins has been recognized (43), and studies with wild type and mutant von Willebrand factor also demonstrate the importance of oligomerization for targeting of von Willebrand factor to lysosome related organelles (44). In the apicomplexan parasite *T. gondii*, a role for the endosome in TgRab51-mediated cholesterol acquisition and transport to the endoplasmic reticulum has been proposed (45), and recently, it was shown that the hexameric micronemal protein complex TgMIC2-M2AP traffics through the trans-Golgi network to the early endosome (21). Although the downstream maturation and trafficking of this complex to the microneme is not yet fully elucidated, exit of the complex from the endosome is dependent upon the N-terminal propeptide sequence of M2AP. Interestingly, EtMIC5 also possesses an N-terminal propeptide sequence that is cleaved *en route* to the microneme (35).

Finally, we can speculate that the EtMIC4–5 complex may be sorted to the microneme by a mechanism similar to that described for proteins of regulated secretory organelles in higher eukaryotes such as granins, which also have a primary acidic structure and calcium binding properties (46). Formation of multimeric complexes of EtMIC4-EtMIC5 could facilitate sorting following aggregation and condensation and could potentially provide a mechanism for the tight packaging for storage of this enormous complex in the confined space of the microneme organelle, which measures only ~100 × 50 nm.

The predicted sequences of EtMIC4 and EtMIC5, which contain TSR, EGF-2, and Apple/PAN domains, suggest the possibility of cell binding activity, and in assays with cultured cells, both EtMIC4 and EtMIC5 were detected in cell-bound fractions. EtMIC5 consists of 11 Apple domains, a module found in the binding regions of factor XI and prekallikrein for which protein-protein interactions have been characterized. Studies in Sarcocystis (47) show that recombinant Apple domains from microneme protein Sm16/17 bind to lactose affinity columns. Other studies in *Eimeria*[5] and *Toxoplasma* (48) show that MIC Apple domains can be purified over lactose affinity columns.

There is an increasing awareness that many biological systems require the formation of complexes composed of many different proteins to function correctly. Thus, in the post-genomic era, the focus of much research is shifting away from large scale identification of individual proteins toward the characterization of functional protein complexes. This is also true for pathogens, and the results presented in this study represent the identification and functional characterization of a novel, high molecular weight protein complex, and therefore, a signif-

[5] F. M. Tomley and J. M. Bumstead, unpublished data.

icant step forward in our understanding of host cell attachment by parasites and the role of protein complexes in general.

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